

Clearance of Influenza Virus Respiratory Infection in Mice Lacking Class I Major Histocompatibility Complex-restricted CD8⁺ T Cells

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Summary

Transgenic mice homozygous for a β_2 -microglobulin (β_2 -m) gene disruption and normal mice that had been treated with a CD8-specific mAb were infected intranasally with an H3N2 influenza A virus. Both groups of CD8 T cell-deficient mice eliminated the virus from the infected respiratory tract. Potent CTL activity was detected in lung lavage populations taken from mice with intact CD8⁺ T cell function, with minimal levels of cytotoxicity being found for inflammatory cells obtained from the antibody-treated and β_2 -m mutant mice. We therefore conclude that cells infected with an influenza A virus can be cleared from the respiratory tract of mice lacking both functional class I major histocompatibility complex (MHC) glycoproteins and class I MHC-restricted, CD8⁺ effector T cells.

The CD4⁺CD8⁺ α/β T cell is considered to be the primary mediator of viral clearance in virus infections (1). The central idea is that the class I MHC glycoproteins that present the viral peptides recognized by the CD8⁺ effectors are widely expressed (or readily induced) in most tissues, thus minimizing the possibility that infected cells will escape immune surveillance (2, 3). The immunopathogenesis of murine influenza pneumonia is thought to be in general accord with this model (4). Potent, influenza-specific class I MHC-restricted CTL activity is found in the lung after 7 d of infection, with virus clearance being only slightly delayed in mice depleted of CD4⁺ T cells (5). Early elimination of influenza virus from naive mice is promoted by the adoptive transfer of virus-specific CD8⁺ effectors, either as cloned lines or as populations of immune spleen cells (4, 6, 7). The immune T cells apparently interact directly with lung cells expressing surface changes induced by the virus, as mice infected simultaneously with two different influenza A viruses only show rapid clearance of the virus for which the transferred, CD8⁺ CTL line is specific (7). However, the conclusion that the CD8⁺ T cell is the sole effector capable of terminating influenza pneumonia has yet to be substantiated. The present analysis thus addresses this question using both conventional mice depleted of CD8⁺ effectors by *in vivo* treatment with a CD8-specific mAb (8, 9), and transgenic mice homozygous for a β_2 -microglobulin (β_2 -m)¹ gene disruption (β_2 -m

[-/-]) that lack functional class I MHC glycoproteins and CD8⁺ α/β T cells (10).

Materials and Methods

Mice. Female C57B1/6J (B6, H-2^b) mice purchased from The Jackson Laboratory (Bar Harbor, ME) were infected at 8 wk of age. Mice transgenic for a homozygous (-/-) β_2 -m gene disruption, and heterozygous (+/-) controls that express class I MHC glycoproteins normally, were derived from (129 \times B6)F2 (H-2^b) founder stock (10). They were bred at the Whitehead Institute, and later transferred to St. Jude Children's Research Hospital. Apart from the influenza infection, mice were maintained under specific pathogen-free conditions.

Viruses. The A/HK \times 31 (H3N2) influenza A virus (11) was grown in the allantoic cavity of embryonated chicken eggs. Stocks of allantoic fluid containing this virus were shown to be free of bacteria, including mycoplasma, and endotoxin (5). The mice were infected intranasally under Avertin (2,2,2-tribromoethanol) anesthesia with 30 μ l of PBS containing 240 hemagglutinating units of the virus, which represents \sim 1/10 of the lethal inoculum for 8-wk, female B6 mice (our unpublished data). Virus was re-isolated from the experimental mice by grinding the lungs in 1.0 ml of PBS, then injecting 200 μ l of serial 10-fold dilutions into the allantoic cavity of embryonated chicken eggs. Samples of allantoic fluid were tested 48 h later for hemagglutinating activity, and the results were reported as the antilog of the dilution at which virus was detected in 50% of the eggs inoculated (12).

Depletion of CD8⁺ T Cells. Mice were depleted of CD8⁺ T cells by intraperitoneal inoculation with 0.5-ml aliquots of a 1:10 dilution of mouse ascitic fluid containing the 2.43.1 mAb (9). The mAb was given 3 d before, on the day of infection, at 3 d after,

¹ Abbreviations used in this paper: β_2 -m (-/-) or (+/-), β_2 -microglobulin homozygous or heterozygous for gene disruption; MLN, mediastinal lymph node.

then at 2-d intervals until the completion of the experiment. The titer of the undiluted ascites was 1:5,000, as determined by flow cytometry after incubation of 150 μ l of ascitic fluid with 10^6 thymocytes (5).

Inflammatory Cells and Lymphocyte Phenotyping. Cells were lavaged from the lungs of 4-10 anesthetized, virus-infected mice as described previously (5). The pooled lung lavage populations were washed, counted, and adhered on plastic for 90 min at 37°C to remove macrophages. The lymphocytes were then processed for flow cytometric analysis using a FACScan® (Becton Dickinson & Co., Mountain View, CA) in single-color or two-color mode. The mAbs used for staining were 2C11 anti-CD3 (13), H57.597 anti- α/β TCR (14), GL3 anti- γ/δ TCR (15), H129.19 anti-CD4 (16), 31M anti-CD8 α (9), and 53-5.8 anti-CD8 β (FITC-conjugated; Pharmingen, San Diego, CA). The first three mAbs were derived from hamsters, while the remainder are of rat origin. The FITC-conjugated second-step reagents for the single-color analysis were goat anti-hamster Ig (Southern Biotechnology, Birmingham, AL) and mouse anti-rat Ig (Jackson ImmunoResearch, West Grove, PA). Binding of the mAbs to CD8 is not blocked by the 2.43.1 mAb used for the *in vivo* depletions.

Cytotoxicity Assays. Lung lavage cells were pooled from 4-10 mice, depleted of macrophages, and tested directly for the presence of cytotoxic T cells. Lymph node cells were first stimulated *in vitro* with a syngeneic, virus-infected alveolar macrophage cell line (17) or splenocytes. Influenza-specific effectors were assayed on MHC-compatible, ^{51}Cr -labeled, A/HKx31-infected, MCS7G (H-2^b) fibroblasts (5) or LB15.13 hybridoma cells (I-A^{d/b}, I-E^{d/b}). The former target detects class I MHC-restricted effectors, while the latter is lysed by both class I and class II MHC-restricted populations. NK cell activity was measured on the YAC-1 target (18). Normal MCS7G or P815 cells were used as targets in the presence of 0.01% PHA lectin (Difco Laboratories, Detroit, MI) to detect any cells with activated lytic machinery. P815 cells with an FcR-bound mAb to CD3e (13) were used to demonstrate the presence

of CD3⁺ cytotoxic effectors. The levels of cytotoxicity are expressed as percent specific ^{51}Cr release in a 6-h assay (5).

Results

***In Vivo* Depletion with a CD8-specific mAb.** Elimination of the CD8⁺ T cells (CD8 α and CD8 β ; Table 1) neither greatly modified the severity of the inflammatory process nor prevented virus clearance (Table 1). The cellular response during infection in normal mice was progressively dominated by the CD8⁺ α/β T cells, with the CD8/CD4 ratios in the lung lavage populations ranging from 2.5:1 on day 5 to >6:1 on day 7. The CD8-depleted mice showed a relative increase in the prevalence of the CD4⁺ subset (Table 1), with the total numbers of CD4⁺ lymphocytes increasing approximately threefold (compared to untreated mice) by 10 d after infection. The numbers of γ/δ T cells were also about twofold higher in mice lacking the CD8⁺ subset. However, we do not know how accurate a reflection the flow cytometry findings are of the total numbers of γ/δ T cells in the lung: recent experiments (our unpublished data) have shown that many cells that express γ/δ TCR mRNA (19) are in a set with high 90° light scatter, which stains nonspecifically and is normally gated out on the flow cytometer.

As might be expected, elimination of the CD8⁺ T cells prevented the development of virus-specific class I MHC-restricted CTL (Table 2). Activation of NK cells, a normal feature of the early response to many infectious viruses (18), was not prolonged in the CD8-depleted mice (YAC-1 target; Table 2). Similarly, the absence of the CD8⁺ α/β T cells throughout the course of the disease process did not facilitate the emergence of other cytotoxic effectors, such as

Table 1. Effect of *In Vivo* Depletion of CD8⁺ T Cells on Virus Clearance and T Cell Localization to the Lung

| Table 1. Effect of In Vivo Depletion of CD8 ⁺ T Cells on Virus Clearance and T Cell Populations | | | | | | | | | | |
|--|----------------------|---------------------|----------------------|-------------------------|------------------------|-------------|-----|-----|----------------|-----------------|
| Group | Days after infection | Virus titer in lung | Cell count/ mouse | Lung lavage populations | | | | | | |
| | | | | Control | Percent cells staining | | | | | |
| | | | | | CD8 α | CD8 β | CD4 | CD3 | α/β | γ/δ |
| | | | $\times 10^5$ | | | | | | | |
| Untreated | 5 | >6.0 | 8 | 3 | 32 | 28 | 13 | 70 | 63 | 13 |
| | 7 | 3.5 | 26 | 1 | 53 | 57 | 10 | 87 | 80 | " |
| | 10 | 0 | 19 | 1 | 67 | 65 | 10 | 93 | 91 | 6 |
| | 13 | 0 | 11 | 1 | 56 | 55 | 16 | 89 | 82 | 7 |
| CD8-depleted | 5 | >6.0 | 10 | 2 | 3 | 1 | 19 | 56 | 41 | 20 |
| | 7 | >6.0 | 20 | 2 | 3 | 2 | 20 | 50 | 44 | 10 |
| | 10 | 0 | 15 | 2 | 2 | 2 | 41 | 77 | 51 | 13 |
| | 13 | 0 | 11 | 1 | 4 | 2 | 38 | 78 | 61 | 12 |

Normal B6 mice and mice depleted of CD8⁺ T cells with the 2.43.1 mAb were infected intranasally with the A/HKx31 virus, (9). The protocols for the *in vivo* T cell depletion, virus titration, and single-color flow cytometry with noncompeting mAbs are all described in Materials and Methods. The control for the flow cytometric analysis was the second antibody alone.

Table 2. Consequences of CD8 Depletion for Virus-specific CTL and NK Cell Activity in Freshly Isolated Lung Lavage Populations

| Days after infection | Group | Percent specific ⁵¹ Cr release (E/T 20:1) | | |
|----------------------|-----------|--|---------------|-------|
| | | MC57G target | | YAC-1 |
| | | With virus | Without virus | |
| 8 | Untreated | 19.2 | 0.1 | 6.0 |
| 6 | CD8- | 3.0 | 2.6 | 18.8 |
| 8 | depleted | 4.5 | 0 | 4.7 |
| 10 | | 1.0 | 0 | 2.9 |

Pooled lung lavage cells from normal and CD8-depleted A/HKx31-infected B6 mice were tested in a 6-h ⁵¹Cr release assay using syngeneic A/HKx31-infected and normal MC57G targets and the YAC-1 NK cell target, as described in Materials and Methods.

CD4⁺ CTL, lymphokine-activated killer cells, or cytotoxic macrophages that could mediate lectin-dependent lysis of P815 target cells (Table 3). The only cytotoxic activity, specific or nonspecific, found for freshly isolated cells was in the lung-lavage populations from influenza-infected mice with intact CD8⁺ T cell function. Lymphocytes taken directly from the regional, mediastinal lymph nodes (MLN) of either the untreated or the CD8-depleted mice were not cytotoxic (Table 3). However, the presence of CTL precursors in MLN from

Table 3. Lectin-dependent Cytotoxicity for Lymph Node and Lung Cells from Normal and CD8-depleted Mice with Influenza

| Source | In vitro culture | E/T ratio | Percent specific ⁵¹ Cr release | | |
|--------|------------------|-----------|---|------------------|--------|
| | | | Untreated | Anti-CD8 treated | |
| | | | day 8 | day 8 | day 10 |
| Lung | Nil | 50:1 | 38 | 1 | 3 |
| MLN | Nil | 50:1 | 1 | 0 | 0 |
| | 5 d | 20:1 | 42 | 0 | 2 |

Some of the A/HKx31-infected B6 mice were treated with the 2.43.1 mAb, and freshly isolated MLN and lung cells were tested for lectin-dependent cytotoxicity in a 6-h ⁵¹Cr release assay. These procedures are described in Materials and Methods. Other cells were cultured for 5 d with an irradiated, influenza-virus infected, class II MHC-positive macrophage cell line of B6 origin in the absence of added IL-2.

the normal (but not the CD8-depleted) mice was shown by culturing for 5 d in the presence of a virus-infected B6 macrophage line (Table 3).

Thus, the development of both CTL in vivo, and precursors that could be expanded in vitro to mediate class I MHC-restricted, virus-specific cytotoxicity, was essentially limited to mice with intact CD8⁺ T cell function. The results establish that influenza virus is cleared effectively in the absence of a substantial population of CD8⁺ effectors. However, the possibility that a few virus-specific CD8⁺ α/β T

Table 4. Virus Clearance and Cytotoxicity in Homozygous (-/-) β_2m Mutant Mice and β_2m Heterozygote (+/-) Control Mice

| Days after infection | Group | Virus isolation from lung | E/T ratio | Percent specific ⁵¹ Cr release* | | | |
|----------------------|-------|---------------------------|-----------|--|-----------------------|-------------|-------|
| | | | | P815 ⁺ α CD3 | P815 ⁺ PHA | Normal P185 | YAC-1 |
| 10 | +/- | 0/4 | 40:1 | 47 | 45 | 3 | 7 |
| | | | 20:1 | 44 | 37 | 0 | 7 |
| | -/- | 1/4 [†] | 40:1 | 9 | 2 | 0 | 0 |
| | | | 20:1 | 7 | 0 | 0 | 0 |
| 13 | +/- | 0/4 | 20:1 | 19 | 31 | 2 | 7 |
| | | | 10:1 | 13 | 30 | 1 | 5 |
| | -/- | 0/4 | 40:1 | 15 | 8 | 1 | 1 |
| | | | 20:1 | 7 | 2 | 0 | 0 |

The mice were infected intranasally with 240 hemagglutinating units of the HKx31 influenza A virus. During the course of the experiment, two of the homozygotes, and one of the controls, died within 16 d of infection. Three remaining β_2m (-/-) mice were surviving 30 d post-infection. The MLN were tested by two-color flow cytometric analysis for the presence of CD8⁺ α/β TCR⁺ lymphocytes, with none being found in the β_2m (-/-) mice.

The results are for lung lavage cells pooled from four mice. No evidence of cytotoxicity was found for cells from the MLN. The assay systems are described in Materials and Methods.

* Virus was detected only in an undiluted lung homogenate of one mouse.

Table 5. Phenotype and Virus-specific Cytotoxicity of $\beta_2\text{-m}$ (+/-) and (-/-) Lymph Node Cells after Stimulation with Influenza-infected B6 Splenocytes

| Group | Percent of cells | | Percent specific ^{51}Cr release (40:1) | | | | |
|-------|--|--|--|--------------------------|---------------------|------------------|-------|
| | CD8 ⁺ α/β ⁺ | CD4 ⁺ α/β ⁺ | LB15.13 With virus | LB15.13 Without virus | MC57G With virus | P815 With CD3 | YAC-1 |
| +/- | 29.6 | 51.6 | 33 | 0 | 41 | 57 | 0 |
| -/- | 0.8 | 70.0 | 22 | 5 | 5 | 33 | 0 |

MLN were obtained from mice infected with A/HKx31 13 d previously. No evidence of cytotoxicity was found for cells tested before in vitro culture. Cells were stimulated with A/HKx31-infected B6 splenocytes for 4 d before assay of cytotoxicity using class I MHC⁺ MC57G and class I and II MHC⁺ LB15.13 cells as targets. The percent of cells was assessed by two-color flow cytometry.

cells have escaped the mAb-depletion regime is difficult to exclude with this experimental system.

Influenza Infection in $\beta_2\text{-m}$ (-/-) Mutant Mice. The $\beta_2\text{-m}$ (-/-) mice lack both CD8⁺ α/β T cells and class I MHC glycoproteins (10). Consequently, it does not seem possible that classical class I MHC restriction (1, 2) could be operating. Influenza virus was substantially cleared from the lungs of both the $\beta_2\text{-m}$ (-/-) mice and the (+/-) controls within 10 d of infection, and completely eliminated within a further 3 d (Table 4). Potent CTL activity mediated by CD3⁺ lymphocytes was present in the lung lavage cells obtained from the (+/-) mice at day 10 after infection, with much lower levels of CD3-dependent (or PHA-dependent) cytotoxicity being observed for inflammatory cells from the $\beta_2\text{-m}$ (-/-) mutants (Table 4). The latter activity could be due to CD4⁺ α/β T cells or γ/δ T cells. Also, there was no indication that the NK response measured on YAC-1 cells was compensating for the lack of the CD8⁺ CTL (Table 4).

Lymph node cells taken from both the $\beta_2\text{-m}$ (+/-) and the (-/-) mice at 13 d after infection were stimulated in vitro with virus-infected splenocytes and assayed 4 d later. No evidence for the development of CD8⁺ α/β T cells was found for the cultures from the (-/-) mice, though effectors lytic for the class II MHC⁺ LB15.13 targets infected with the HKx31 virus were detected (Table 5). Cultures from the (+/-) controls contained a predominance of CD8⁺ α/β T cells, showed strong class I MHC-restricted, virus-specific CTL activity, and a higher level of CD3-dependent lysis than that observed for the (-/-) mice lacking the CD8⁺ population. Thus, while virus-specific, class II MHC-restricted CTL can be generated in vitro from the $\beta_2\text{-m}$ (-/-) mice, the overall level of cytotoxic effector function is lower than that found for the (+/-) controls.

Discussion

The present experiments show that mice lacking functional class I MHC glycoproteins and/or CD8⁺ class I MHC-restricted effector T cells can eliminate influenza virus from the murine lung. The role of CD4⁺ T cells in murine

influenza pneumonia has been explored previously by transferring immune effectors into naive, virus-infected mice: bulk populations of CD4⁺8⁺ T cells isolated directly from spleens of virus-primed mice did not clear influenza virus (4), and even caused enhanced immunopathology, while in vitro cultured virus-specific CD4⁺8⁺ T cell clones promoted rapid recovery (20, 21). Also, removing the CD4⁺ population by in vivo treatment with mAbs caused little delay in virus clearance, though the virus-specific IgG response was greatly diminished (5, 22). The overall conclusion is that this infection can be terminated by either CD4⁺ or CD8⁺ effectors, but simultaneous removal of both these T cell subsets leads to death (our unpublished data). However, the response may normally be skewed (4) to emphasize the development of CD8⁺ T cell-mediated immunity.

The fact that elimination of the CD4⁺ and CD8⁺ T cell subsets by in vivo treatment with mAbs is lethal for mice with influenza (our unpublished data) indicates that CD4⁺8⁺ γ/δ T cells acting alone cannot compensate for the absence of the CD4⁺ and CD8⁺ effectors. Cells expressing γ/δ TCR mRNA are prominent late in the course of influenza pneumonia, but the frequency of lymphocytes that express a functional γ/δ TCR within this population has not yet been established (19). It is clear that the majority of the γ/δ TCR mRNA⁺ cells found in the lungs of normal mice with influenza are CD4⁺8⁺, α/β TCR⁺, and are not phagocytic (19, 23). Uninfected $\beta_2\text{-m}$ (-/-) transgenic mice show normal numbers of γ/δ T cells (10), and there is no evidence that these lymphocytes are functionally defective. However, whether or not the γ/δ T cells play any role in virus clearance is yet to be established.

Cells from the $\beta_2\text{-m}$ (-/-) mice do express low concentration of cell surface H-2D^b glycoprotein, and in the presence of exogenous bovine $\beta_2\text{-m}$, act to some extent as targets for alloreactive CTL specific for H-2D^b (10). The H-2D^b molecule presents the influenza virus nucleoprotein peptide recognized by most of the CD8⁺ T cells generated during the response to this virus in H-2^b mice (3). The inability to generate class I MHC-restricted CTL activity in B6 mice infected with an influenza A virus establishes again (10) that,

in the absence of endogenous $\beta_2\text{-m}$ protein, the H-2D^b molecule is nonfunctional in vivo.

This study establishes that, while class I MHC-restricted CD8⁺ CTL may be the main mediators of influenza virus clearance in normal mice (1, 4, 6, 7), an alternative mechanism exists for terminating the infectious process. The likely effector is the virus-specific, class II MHC-restricted, CD4⁺ T cell (20, 21). These CD4⁺ T cells could mediate virus clearance by various mechanisms, such as promoting the local

influenza-specific B cell response (21), acting directly on class II MHC⁺ target cells in the virus-infected lung, or providing help for other potential effectors such as the γ/δ T cells. The results indicate a redundancy in cell-mediated effector mechanisms, at least in young, healthy adult mice infected with a virus that grows mainly in surface epithelium.

We thank Anthony McMickle, Mohammad Mehrpooya, and Janet M. Loring for capable technical assistance, and Jim Houston and Betsy Sidell for help with the flow cytometry.

This study was supported by research grants CA-21765 and AI-29579 from the United States Public Health Service, and by the American Lebanese Syrian Associated Charities. M. Zijlstra is supported by a Fan Fox & Leslie R. Samuels Foundation Fellowship from the Cancer Research Institute.

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Received for publication 10 July 1991.

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